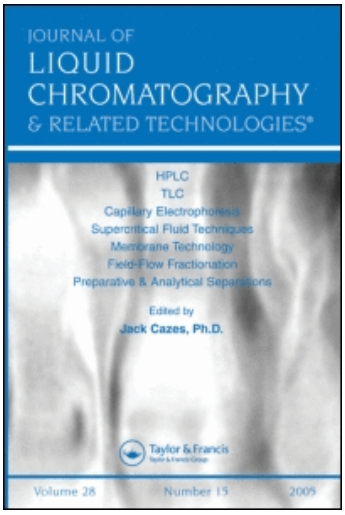

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ISOLATION OF THYMOSIN α_1 FROM THYMOSIN FRACTION 5 BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An HPLC system for quickly isolating individual peptides from thymus extract is described. A series of reversed phase separations followed by ion-exchange HPLC produces pure peptides with minimal chromatographic time. A 2.4 gram column load of crude extract was carried through to the isolation of 4 mg of pure thymosin α_1 within 6-8 hours of chromatography.

INTRODUCTION

The purification of peptides on a preparative scale by high performance liquid chromatography (HPLC) has been the subject of many papers (1) and (2). However, most preparative runs involve only a few milligrams of material. For the past two years, we have been purifying synthetic peptides on a 10 mg to 2 g scale by reversed phase HPLC on a routine basis (3).

The commercial availability of larger columns permitted increases in column loads to more than 2 grams, even for complex mixtures. We have applied the technique to the isolation of individual components from the complex mixture of peptides present in calf thymus extract (Fraction 5) prepared according to the procedure of Goldstein et al. (4).

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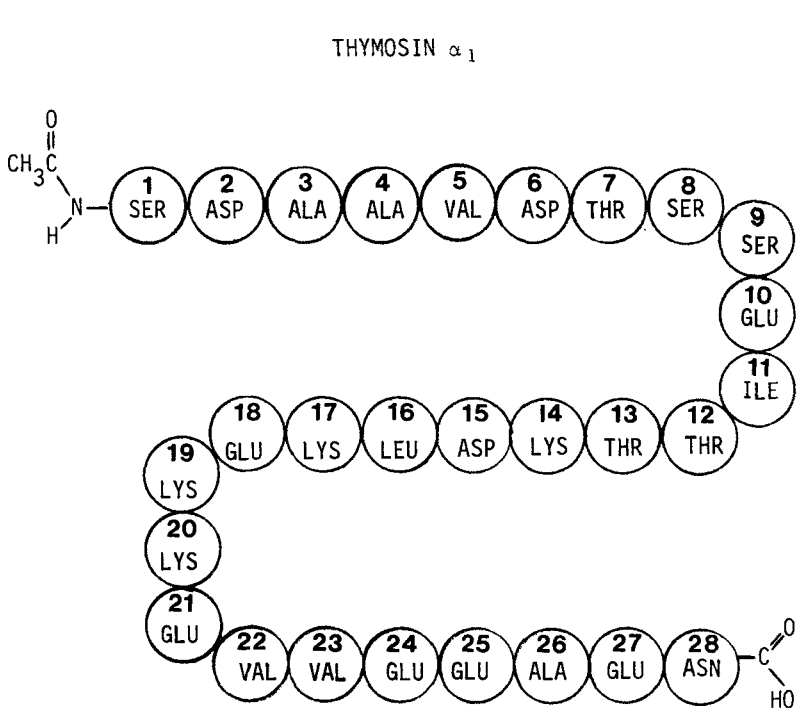

FIGURE 1. The structure of Thymosin α_1, molecular weight 3,108 daltons, as reported by Goldstein (4).

The speed and high resolution of HPLC, used in conjunction with volatile pyridine-acetic acid buffer systems, are advantageous in the isolation of naturally occuring peptides. The combination of reversed phase techniques with high performance ion exchange technology leads to a system of very high resolution. Initial separation by size, followed by separation based on lipophilicity at 2 different pH settings, and a final ion exchange step at neutral pH produces individual isolated target peptides quickly. The application of this procedure to the isolation of homogeneous thymosin α_1 (Fig. 1) from thymosin fraction 5 is described herein.

MATERIALS AND METHODS

The thymus extract was prepared according to the procedure of Goldstein et al. (4) and stored as a frozen solution in 0.1M ammonium bicarbonate, pH 7. A peptide content of 68 mg/mL was determined with the Bio-Rad Protein Assay (5).

A Laboratory Data Control gradient elution HPLC system was used in this work. Columns were purchased from the manufacturers: Waters Associates Inc. (μ Bondapak C18, C18 Sep-Pak), Whatman Inc. (Partisil SAX, ODS-2), and E.S. Industries (C-8). Peptides were detected by automatically removing a 25 μL aliquot of the column effluent, reacting it with o-phthalaldehyde (OPA) in pH 10.5 borate buffer, and monitoring the fluorescent product with a Varian Fluorochrome monitor (6). Alternatively, peptides were detected by monitoring the 206 nm absorbtion of the column effluent with a Laboratory Data Control Spectromonitor III.

All solvents were of Burdick and Jackson Distilled in Glass grade; water was purified via a Hydro Service system. Chromatography was carried out at ambient temperature, generally 19-22°C.

Amino acid analyses were carried out on samples digested with 6N HCl-propionic acid at 150°C for 15 minutes (7) and analyzed on a Dionex D-300 Amino Acid Analyzer.

Direct Isolation of Thymosin α_1 Peptide from Thymosin Fraction 5:

A solution of thymosin fraction 5 in 0.1 M NH_4HCO_3, pH ~ 7.5, containing 68 mg peptide per mL was filtered through a 0.8μ Millipore-membrane. Thirty-five mL of filtrate, corresponding to 2.38 g of peptides were pumped onto a 3/4" x 12" column of 10μ C-8 packing (E.S. Industries) at 1 mL/minute. The column was then eluted with 8% pyridine-3% acetic acid in water at 3 mL/minute. Fractions were collected at 1 minute intervals, with a 25 μL aliquot being removed

for OPA derivatization every time the fraction collector indexed. The fluorometer output was recorded.

The fractions that contained thymosin α_1 were delineated by injecting 50 µL aliquots of each fraction onto a calibrated 0.46 x 30 cm E.S. Industries 10µ C-8 column and eluting with 8% pyridine-2.6% acetic acid in water at 2 mL/minute. This column was also monitored by OPA derivatization. After all the thymosin α_1 had been eluted, the large column was "stripped" with a linear gradient from starting buffer to 90% of 1:1 acetonitrile-isopropanol containing 8% pyridine-2.6% acetic acid over 60 minutes. Pools were made of fractions with similar composition, and then lyophilized. A total of 96 mg of material containing thymosin α_1 in 4 pools was obtained.

Twenty milligrams of the richest pool was chromatographed on a Whatman ODS-2 column, 0.96 x 50 cm with a linear gradient of 10-50% CH_3CN in 0.022% trifluoroacetic (TFA) acid over 80 minutes at 3 mL/min; monitored at 206 nm (Fig. 2). One minute fractions were collected. Aliquots of the major peak were injected onto an analytical column, µ Bondapak C18, eluted with 18% CH_3CN in 0.022% TFA. Those fractions richest in α_1 were pooled, concentrated in vacuo at 40°C to remove CH_3CN and lyophilized. The 11.2 mg of material obtained were chromatographed on a Whatman Partisil SAX column, 0.9 x 50 cm; using a linear gradient of 0.045 to 0.075 M KH_2PO_4, pH 6.5, over 60 min. at 3 mL/min., with the 206 nm absorption being monitored (Fig. 3). One minute fractions were collected. Again, aliquots of major peaks were monitored by analytical HPLC.

Those fractions consisting of homogeneous α_1 were desalted on a C18 Sep-Pak cartridge (Waters Associates), and lyophilized to produce 2.2 mg of thymosin α_1. The product was homogeneous by analytical HPLC. Amino acid analysis: Asp 4.2 (4), Thr 2.7 (3), Ser 2.7 (3), Glu 6 (6), Ala 3.0 (3), Val 2.2 (3), Ile 1.1 (1), Leu 1.1

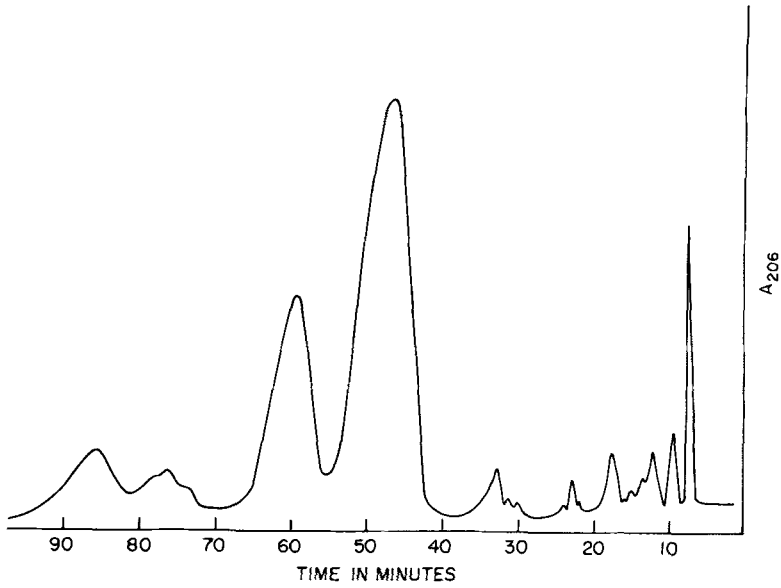

FIGURE 2. Chromatography of thymosin α_1 region fraction pools on Partisil ODS-2 column, 0.9 x 50 cm, in acetonitrile-0.022% trifluoroacetic acid, 10% to 50% over 80 minutes at 3 mL/min. as indicated by X-X-X. Solid line is the 206 nanometer absorbtion.

(1), Lys 4.2 (4). The low value for valine is due to the known resistance of the Val-Val bond to hydrolytic cleavage.

The 2.2 mg of product represents only a fraction of the thymosin α_1 content of the starting thymosin fraction 5. The α_1 tends to be distributed among several adjacent pools, possibly due to its tendency to aggregate. Re-chromatography of other thymosin α_1 containing pools produced an additional 2 mg of product.

On a real time basis, about 6-8 hours were required for the separation, exclusive of the time required for lyophilization. This represents a saving in time and effort over the conventional chromatography which requires 2 cellulose ion exchange steps and 2 Sephadex G-10 desalting steps, consuming about 5 days.

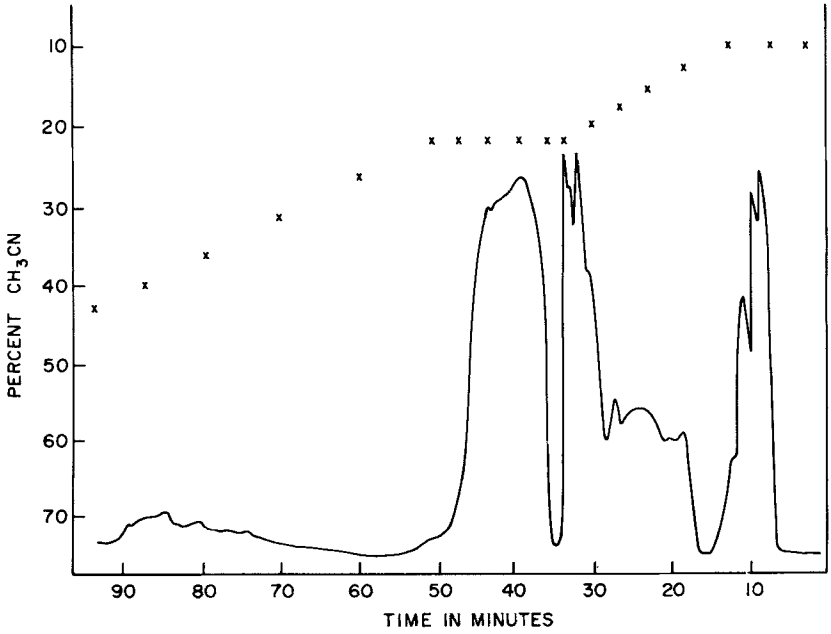

FIGURE 3. Ion exchange chromatography of thymosin α_1 peak pool from Figure 2 on a Partisil SAX column, 0.9 x 50 cm, in linear gradient of 0.045 to 0.075 M potassium phosphate pH 6.5, over 60 min. at 3mL/min.; 206 nanometer absorbtion is shown.

The use of the C18 Sep-Pak cartridges for the desalting of the ion exchange column eluate represents a substantial saving in itself. Individual fractions were pushed through a washed cartridge (50% CH_3CN in 0.022% TFA 5mL, 0.022% TFA 5 mL) with a syringe. After all the fractions to be pooled had been loaded, the salt was rinsed out with 5 mL H_2O, and the peptide removed by 3 successive washings with 50% CH_3CN in H_2O (3 mL, 2 mL, 1 mL). HPLC analysis of the last wash showed only a trace of peptide. After concentration in vacuo to remove the CH_3CN, the wash was lyophilized. The entire process requires only an hour or two.

TABLE 1
Amino Acid Peptides from Thymosin Fraction 5

	R-2	R-3	R-4	R-5	α_1
Asp	6.6	2.6	3.9	5.5	4
Thr	4.1	1.6	2.0	3.0	3
Ser	4.1	2.4	2.5	3.2	3
Glu	1.9	4.7	5.9	7.0	6
Pro	---	---	---	1.5	-
Gly	---	1.3	1.1	1.3	-
Ala	3.6	1.6	3.4	.8	3
Val	3.0	2.1	1.9	2.4	3
Ile	1.0	.9	1.0	1.0	1
Leu	1.0	1.1	1.0	1.0	1
Tyr	---	.8			-
Lys	6	3	3.8	3.7	4

Other peptides were isolated during the course of this separation, including several normally present as contaminants in thymosin α_1 isolated by open column methods. Table I shows the amino acid composition of 4 such contaminants which were found to co-elute with thymosin α_1.

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